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Short Communication

Tentative Colistin Epidemiological Cut-Off Value for *Salmonella* spp.

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Abstract

The objective of this research was to determine minimal inhibitory concentration (MIC) population distributions for colistin for *Salmonella* on subtype level. Furthermore, we wanted to determine if differences in MIC for colistin could be explained by mutations in *pmrA* or *pmrB* encoding proteins involved in processes that influence the binding of colistin to the cell membrane. During 2008–2011, 6,583 *Salmonella enterica* subsp. *enterica* isolates of human origin and 1931 isolates of animal/meat origin were collected. The isolates were serotyped, and susceptibility was tested towards colistin (range 1–16 mg/L). Moreover, 37 isolates were tested for mutations in *pmrA* and *pmrB* by polymerase chain reaction (PCR) and DNA sequencing. MIC distribution for colistin at serotype level showed that *Salmonella* Dublin ($n = 198$) followed by *Salmonella* Enteritidis ($n = 1247$) were less susceptible than "other" *Salmonella* serotypes originating from humans ($n = 5{,}274$) and *Salmonella* Typhimurium of animal/meat origin ($n = 1794$). MIC was ≤ 1 mg/L for 98.9% of "other" *Salmonella* serotypes originating from humans, 99.4% of *Salmonella* Typhimurium, 61.3% of *Salmonella* Enteritidis, and 12.1% of *Salmonella* Dublin isolates. Interestingly, *Salmonella* Dublin and *Salmonella* Enteritidis belong to the same O-group (O:1, 9,12), suggesting that surface lipopolysaccharides (LPS) of the cell (O-antigen) play a role in colistin susceptibility. The epidemiological cut-off value of >2 mg/L for colistin suggested by European Committee on Antimicrobial Susceptibility Testing (EUCAST) is placed inside the distribution for both *Salmonella* Dublin and *Salmonella* Enteritidis. All tested *Salmonella* Dublin isolates, regardless of MIC colistin value, had identical *pmrA* and *pmrB* sequences. Missense mutations were found only in *pmrA* in one *Salmonella* Reading and in *pmrB* in one *Salmonella* Concord isolate, both with MIC of ≤ 1 for colistin. In conclusion, our study indicates that missense mutations are not necessarily involved in increased MICs for colistin. Increased MICs for colistin seemed to be linked to specific serotypes (*Salmonella* Dublin and *Salmonella* Enteritidis). We recommend that *Salmonella* with MIC of >2 mg/L for colistin be evaluated on the serovar level.

Introduction

The interpretive criteria are crucial when determining bacteria as either resistant or sensitive to an antimicrobial drug. The European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2011) presents a clinical breakpoint and an epidemiological cut-off value of >2 mg/L for colistin, based on a few isolates, whereas *Salmonella* isolates with a Minimal Inhibitory Concentration (MIC) of ≤ 2 mg/L should be reported as sensitive or wildtype. Clinical Laboratory Standards Institute (CLSI) defines clinical breakpoints including also treatment efficacy (CLSI, 2011). The epidemiological cut-off value is defined based on MIC population distributions of *Salmonella* spp. (EUCAST, 2011).

Previously, colistin was used less frequently due to toxicity, but recently it has become the first choice treatment of severe infections involving some multidrug resistant Gram-negative bacteria (Lim *et al.*, 2010).

We hypothesized that colistin MIC population distributions of *Salmonella* on serotype level differ and MICs of colistin are increased in *Salmonella* isolates with missense mutations in *pmrA* or *pmrB* encoding products involved in processes that influence cell membrane binding of colistin (Roland *et al.*, 1993; Sun *et al.*, 2009).

The objective of this work was to determine MIC population distributions for *Salmonella* on subtype level and to determine if differences in MIC could be explained by mutations in *pmrA* or *pmrB*.

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Methods

From 2008 to 2011, the MIC for colistin (concentration range 1–16 mg/L) was determined using commercially dehydrated antimicrobial agents in microtiter wells (Sensititre™; TREK Diagnostic Systems Ltd., West Sussex, United Kingdom) as part of the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) (CLSI, 2008; DANMAP, 2008–2010). From human patients in Denmark, 6,583 *Salmonella enterica* subsp. *enterica* isolates were tested. Among these isolates, the most common serotypes were *Salmonella* Typhimurium (3,281 isolates, including monophasic variants *S*. 4,5,12:i:- and *S*. 4,12:i:-), *Salmonella* Enteritidis (1,178 isolates), and *Salmonella* Dublin (131 isolates). The remaining 1,993 isolates belonged to 198 different serotypes. Furthermore, 1,795 *Salmonella* Typhimurium, 69 *Salmonella* Enteritidis, and 67 *Salmonella* Dublin from food animals and raw meat were tested.

In order to determine if differences in MIC were caused by mutations in *pmrA* and *pmrB*, we looked for mutations in 37 isolates (with MIC and identification verified) of serovar *Salmonella* Dublin ($n=22$), *Salmonella* Enteritidis ($n=14$), and *Salmonella* Typhimurium ($n=1$) from human infections in 2009 with MIC values of 2, 4, or 8 mg/L. In addition, one isolate of each of the above-mentioned three serotypes, plus one *Salmonella* Concord and one *Salmonella* Reading with MIC of ≤1 were included to look for differences in susceptible isolates of five different serotypes. The *pmrA* and *pmrB* genes were sequenced using the primers reported by Sun *et al*. (2009) and additional new primers designed to fully include *pmrB* (forward: 5′-CGACGGACTCAATCTCAAG-3′; reverse: 5′-GCATATACATAATTTGCGCGA-3′).

Results and Discussion

The MIC distribution for all *Salmonella* isolates is shown in Figure 1A; the MIC distributions for *Salmonella* Dublin, *Salmonella* Enteritidis, *Salmonella* Typhimurium (food animals and raw meats), and "other" serotypes originating from humans are presented in Figure 1B. *Salmonella* Dublin followed by *Salmonella* Enteritidis were less susceptible than the other *Salmonella* serotypes. Interestingly, *Salmonella* Dublin and *Salmonella* Enteritidis have the same O-antigen formula (O:1, 9,12).

Therefore, we also looked at the MIC distribution of colistin for available *Salmonella* Napoli ($n=18$) and *Salmonella* Panama ($n=30$), which also have this O-antigen formula. Although these two serovars also seemed less susceptible to colistin than *Salmonella* in general, the trend was not as pronounced; MIC was ≤1 mg/L for 98.9% of "other" *Salmonella* serotypes of human origin, 99.4% for *Salmonella* Typhimurium from animal/meat origin, 89% for *Salmonella* Napoli, 80% for *Salmonella* Panama, 61.3% for *Salmonella* Enteritidis, and 12.1% for *Salmonella* Dublin. This suggests that the surface lipopolysaccharides (LPS) of the cell (O-antigen) play a role in colistin susceptibility. The epidemiological cut-off value suggested by EUCAST (>2 mg/L) is placed inside the distribution for both *Salmonella* Dublin and *Salmonella* Enteritidis. Due to the low number of especially *Salmonella* Dublin when compared to other *Salmonella*, these differences related to serotypes are overlooked when merging all *Salmonella* into the same distribution (Fig. 1).

Colistin targets the cell envelope, and the interaction between the cationic polypeptide and negatively charged LPS

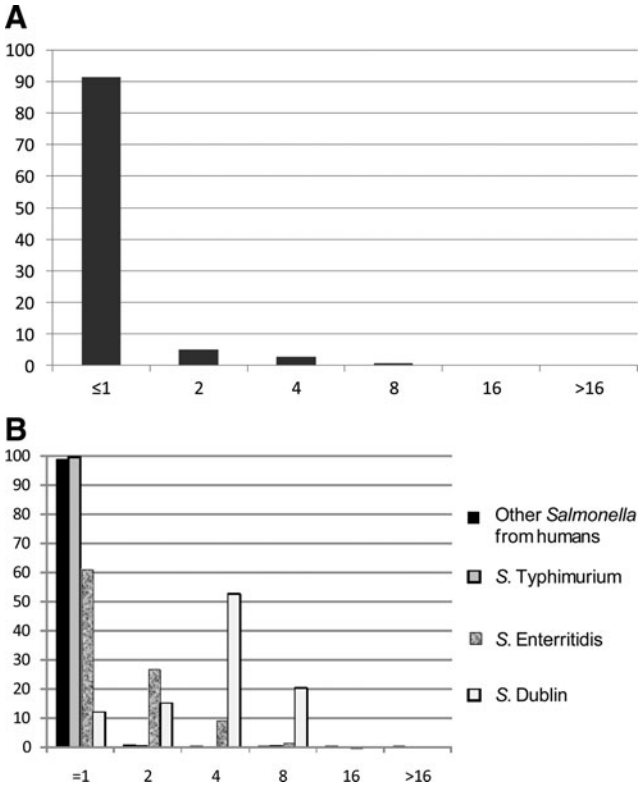

FIG. 1. **(A)** Percentage distribution of minimal inhibitory concentration (MIC) of colistin determined for all *Salmonella* serovars analyzed ($n=8,514$). **(B)** MIC distribution of "other" *Salmonella* serovars from humans ($n=5,274$), *Salmonella* Typhimurium from food animals and raw meats ($n=1,795$), *Salmonella* Enteritidis ($n=1,247$), and *Salmonella* Dublin ($n=198$).

leads to a disturbance of the outer membrane and a subsequent increase in the permeability of the cell envelope resulting in death (Schindler and Osborn, 1979; Sun *et al*., 2009). Increase in MIC for *Salmonella* Typhimurium has been mapped to *pmrA* and *pmrB*, which constitute a two-component regulatory system. PmrB is a sensor histidine kinase, and PmrA is the cognate response regulator. The modifications in these genes influence the regulation of proteins, which makes the LPS less negatively charged and thereby decrease the binding of colistin (Roland *et al*., 1993; Sun *et al*., 2009).

Sun *et al*. (2009) found 27 missense mutations in *pmrB* and *pmrA* that lead to an increased MIC to colistin of up to 4.4 mg/L. For the isolates tested in the present study, all *Salmonella* Dublin isolates, regardless of the MIC value, had identical *pmrA* and *pmrB* sequences. Compared to the *Salmonella* LT2 strain (GenBank accession no. NC003197), the *Salmonella* Dublin isolates had a point mutation in *pmrB* position 25 (A→C), leading to an amino acid change in position 9 from threonine to proline. None of the other isolates had this mutation. In addition, five and six synonymous point mutations were seen in *pmrB Salmonella* Dublin and *Salmonella* Enteritidis isolates, respectively (two of them were identical, positions 492 and 750). No missense mutation was present in *pmrA*, but synonymous point mutations were seen at positions 177 and 322 in *Salmonella* Dublin (C→T) and at positions 322 and 414 in *Salmonella* Enteritidis (C→T). In addition, missense

mutations in *pmrA* were found in one *Salmonella* Reading isolate, and a missense mutation in *pmrB* was found in one *Salmonella* Concord isolate; both had MIC of ≤1. Therefore, missense mutations are not necessarily involved in increased MICs for colistin.

In conclusion, since increased MIC seemed linked to serotype, we recommend that isolates with MIC of >2 mg/L for colistin in *Salmonella* spp. be evaluated on the serotype level. More detailed data when making population distributions (such as interpreting results based on the subtype level and looking for resistance mechanisms) may be necessary when determining interpretive criteria.

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Disclosure Statement

No competing financial interests exist.

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